

# Cryopreservation and Genetic Stability Assessment of Threatened Medicinal Plant (*Ziziphora tenuior* L.) Grown Wild in Jordan

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## Abstract

*Ziziphora tenuior* L. is one of the important medicinal plants that belong to the *Lamiaceae* family. It is a rare species with a promising medicinal potential and grows wild in the southern part of Jordan. Unfortunately, this plant might be totally extinct from the wild due to over-exploitation. Two cryopreservation techniques (encapsulation-dehydration and encapsulation- vitrification) were applied for *in vitro* conservation of this valuable medicinal plant, and after that the explants were tested for their genetic stability using Amplified Fragment Length Polymorphism (AFLP) technique. In the encapsulation-dehydration experiment, the results revealed that 40% of the cryopreserved shoot tips survived when they were dehydrated chemically on 0.75 M sucrose in MS supplemented media for one day and exposed to air dehydration for 6 hrs. Moreover, the best recovery rate (20%) was obtained when either 0.5 M or 0.75 M sucrose MS supplemented media were used as preculture media for the shoot tips for one day, followed by air dehydration for 4 or 6 hrs. Meanwhile, in the encapsulation -vitrification experiment, the highest survival (37.5%) and recovery (10%) percentages of the cryopreserved shoot tips were obtained when the encapsulated shoot tips were pretreated for 60 min. with the loading solution before being exposed to PVS2 vitrification solution and LN. AFLP technique had clearly showed that, there were no genetic variations between the shoot tips of *Ziziphora tenuior* L., before and after cryopreservation.

**Keywords:** Cryopreservation, Encapsulation-dehydration, Encapsulation- vitrification, *Ziziphora tenuior* L.

## 1. Introduction

*Ziziphora tenuior* L. is one of the important species that belong to the family Lamiaceae. It is widely spread in many countries around the world, such as China, Iran, Afghanistan and Iraq (Zargari, 1995). *Ziziphora tenuior* L. is also distributed in Jordan in Steppes, Al-Nagab desert, lower Jordan valley, Araba valley, Amman, Madaba and Edom (Zohary and Feinbrun, 1978). Also, according to Al-Rawashdeh, (2011), *Ziziphora tenuior* L. is spread particularly in the southern part of Jordan mainly at Alshoubak region (Al-Rawashdeh, 2011). This plant is distinguished for its valuable medicinal potential as it has been used in folk medicine in many treatments (Ozturk et al., 1995). For example, it was used for the treatment of dysentery, fever, diarrhea, coughing, bladder stone, painful menstruation, stomach tonic and abortifacient

(Naghbi et al., 2005). Furthermore, it is used in making herbal tea due to its odors (Al-Rawashdeh , 2011). In Jordan, few studies had researched *Ziziphora tenuior*. For example, *Ziziphora tenuior* was undergone molecular taxonomy using RAPD technique, and the result of this study confirmed that there is a variation between this plant and *Mentha* sp. (Al-Rawashdeh, 2011). The other study that highlighted *Ziziphora tenuior* was conducted by Oran (2013) who searched for the flowering plants that grow wild in Tafila Province, and reported that this genetic resource is under threats of extinction. This was due to several reasons including illegal collection, agricultural expansion and urbanization, deforestation, overgrazing, soil erosion, and depletion of water resources (Oran, 2013). So, there is a great need for protecting and conserving this important genetic resource to insure its availability for scientific research and phytoindustry.

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Biotechnology is an integral part of international plant conservation programs and plays a role in preserving plant genetic resources. Cryopreservation is a part of biotechnology (Bajaj, 1995; Benson, 1999) and during the last 25 years, cryopreservation was proved to be the most precious method for biological materials long-term conservation. Cryopreservation is simple and applicable to a wide range of genotypes (Engelmann, 2004). Cryopreservation is considered safe, cost-effective and strategy for long-term preservation of biological materials (Reed, 2008; Shibli et al., 1999b). Among the advantages of cryopreservation are that it has low maintenance cost, allows small storage space requirement and there is no need for many replicates to conserve a plant (Shibli and Al-Juboory, 2000). Moreover, cryopreservation has been used for storing many dissimilar types of plant material, such as embryonic axes isolated from seeds, seeds, and vegetative propagated plant material, including pollen, apical or auxiliary buds, embryogenic tissues and somatic embryos (Salaj et al., 2011; Teresa et al., 2010). Hypothetically, using cryopreservation storage plant materials could be preserved without changes for unlimited periods (Wen and Wang, 2010).

Regeneration and genetic stability potential of cryopreserved plant materials are maintained for an indefinite period (Al-Ababneh et al., 2003). Problems such as genetic instability and risk of loss biological materials due to contamination or human error during subculture can be overcome (Kaviani, 2011). Due to the possibility of presence of epigenetic alterations after cryopreservation, there was a need to validate plant genetic uniformity of the cryopreserved plant material after exposure to LN (Harding, 2004; Micula et al., 2011). To detect any possible genetic variation in plants material, several molecular techniques have been applied (Harding, 2004). DNA (RAPD) and amplified fragment length polymorphisms (AFLP) were the most popular markers used in this domain (Micula, et al., 2011).

Amplified fragment length polymorphisms (AFLP) technique is widely used as a DNA fingerprinting system for detecting genetic variation among living organisms (Micula, et al., 2011). This technique is distinguished for its high reproducibility and high levels of polymorphism (Hao et al. 2001; Nighat et al., 2010). In addition to the ability of AFLP to permit the simultaneous analysis of many loci spread over the entire genome without need for a prior knowledge of the organisms under study, AFLP proved to be a very sensitive, reliable fingerprinting technique that can be applied to resolve differences between isolates of the same species in a broad range of taxa in most living organisms (Muller et al., 2007).

There is no reported literature on the in vitro conservation or genetic assessment of *Ziziphora tenuior* L. which grows wild in Jordan. Therefore, this research was conducted to study the possibility of long-term conservation (cryopreservation) of *Ziziphora tenuior* L. using encapsulation-dehydration and encapsulation-vitrification methods and to test genetic stability of cryopreserved plant material using AFLP technique.

## 2. Materials and Methods

### 2.1. Establishing Mother Stock

*Ziziphora tenuior* L. seeds were originally collected from Al-Hisha, Al-Shoubak region (30° 31' 53" N, 35° 33' 39" E) southern Jordan. Surface-sterilization of seeds was done by dipping them in 70% ethanol solution for 30s, before soaking in a 20% aqueous solution of 5.4% sodium hypochlorite for 20 min with gentle shaking, followed by three washes with sterilized distilled water under laminar air-flow cabinet. After that, the sterilized seeds were inoculated into test tubes containing Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) without growth regulators. Cultures were left in the growth room conditions in dark at 25±1°C until full germination of seeds. Germinated seedlings were transferred to normal conditions of growth room and maintained under daily light regime of 16-h (photosynthetic photon flux density (PPFD) = 40-45 μ mol. m<sup>-2</sup> sec<sup>-1</sup>) light, 8-h dark at 24 ± 1°C. Cultures were sub-cultured periodically every (4-5weeks) to MS hormone free (HF) medium for mother stock establishment.

### 2.2. Encapsulation-Dehydration

For shoot tip encapsulation, two different liquid media were prepared from stock solutions, the first medium contained 3% sodium alginate and calcium free-MS. The second medium was prepared by using Murashige and Skoog media that includes (100 mM) of calcium (CaCl<sub>2</sub>) with of final concentration. Next, *Ziziphora tenuior* L. shoot-tips were dissected in vitro and precultured 3 days on solid MS-HF (hormone free) medium provided with 0.3 M sucrose in dark at growth room conditions. Shoot tips were taken individually by using 1ml sterile micropipette with some alginate solution, and then were soaked into liquid MS-HF medium provided with 100 mM CaCl<sub>2</sub> and 0.3 M sucrose to produce beads that had a diameter of about 5 mm. Some produced beads had to be without shoot tips for moisture content determination and all the beads had to be polymerized for 30 min with stirring.

Produced beads were then transferred to MS-HF liquid medium containing 0.5 and 0.75M sucrose and lightly rotated on a shaker for 1 to 3 days, after that the media containing 0.5M or 0.75M sucrose was removed and beads dehydrated under laminar air-flow cabinet on a sterilized filter paper for 0, 2, 4, and 6 hrs, then half of the beads were transferred to 2 ml sterile cryovials and dipped into liquid nitrogen for at least 1 hr at (-196°C). Next, the cryovials were thawed in a water bath at 38-40°C for 2-3 min, while the other half of beads was not exposed to liquid nitrogen. Both halves were transferred to a recovery MS media provided with 0.1 M sucrose and preserved for 7 days in dark for growth, and next transferred to normal growth conditions for 7 days. After that, shoot tips were studied for 4 weeks for survival and recovery signs. Survival percentage of cryopreserved or non-cryopreserved shoot tips was determined by using TTC (2, 3, 5-triphenyl tetrazolium chloride) for each treatment, where 50 *Ziziphora tenuior* L. shoot tips from each treatment were placed in 5 cryovials (10 shoot

tips/cryovial) and 1 ml of Tetrazolium (TTC) salt solution 0.5% (w/v) was added to each cryovial and kept in a complete darkness for 16 hours. Survival percentage = (number of red shoots / total number of shoots) × 100%

The presence of color with pink or red in the survived shoot tip is a result of reducing Tetrazolium (TTC) salt solution to formazan using hydrogen ions released from viable plant cells as a result of respiration.

For the determination of beads moisture content, after each dehydration period, a fresh weight of beads was measured; the beads were dried at 80 °C in an oven for 16 hrs and then reweighed. Moisture Content (MC) was determined by the formula:

$$MC \% = \frac{(\text{Beads fresh weight} - \text{Beads dry weight})}{\text{Beads fresh weight}} \times 100.$$

### 2.3. Encapsulation-Vitrification

Encapsulation of *Ziziphora tenuior* L. shoot-tips in beads was done as mentioned in the encapsulation method previously. The beads were placed in sterilized cryovials for different periods (10, 20, 40, 60 and 80 min) with the loading solution (0.4 M sucrose and 2 M glycerol in HF-liquid MS medium). Next, the loading solution was replaced with full strength plant vitrification solution 2 (PVS2) consisted of 15% ethylene glycol, 30% glycerol and 15% DMSO (w/v) in a hormone free liquid medium provided with 0.4 M sucrose for 20 min at room temperature.

Half of the cryovials was not exposed to (LN) and the other half of the cryovials was soaked in (LN) and stored for at least 1 hr. then they were thawed at 38-40 °C for 2-3 min. after that the PVS2 solution in both halves (with and without LN) was replaced by unloading solution and washed three times for 30 min. Other testing procedures were done as in the experiment described above.

### 2.4. Genetic Stability Assessment of *Ziziphora tenuior* L. using Amplified Fragment Length Polymorphism AFLP technique

Five seeds of *Ziziphora tenuior* L. were collected randomly before the seeds were cultured separately on MS-FH media for full germination in the growth room chamber conditions (as mentioned in mother stock establishment section). After that aseptically germinated seedlings were propagated separately for each seed. Next each seed had its mother stock shoots alone. The encapsulation-dehydration technique was applied for cryopreservation of each seed mother stock shoot tips individually. After that, a sample of each seed mother stock was taken before and after they were cryopreserved to test their genetic stability using AFLP technique as described by (Vos et al., 1995).

#### 2.4.1. DNA Extraction

Two grams of *Ziziphora tenuior* L. plant samples were grinded in liquid nitrogen in 1.5 ml tube. Then about 40 mg of tissues powder were placed in 1.5 ml and 600 µl of Nuclei Lysis solution was added. The solution was then vortexed and then incubated at 65 °C for 15 min. RNase solution 3 µL was added to the cell lysate, and the samples were mixed by inverting the tube two to five times and incubated for 15 min at 37 °C.

Samples were left at room temperature for cooling. Then protein precipitation solution (200µl) was added and vortexed vigorously at high speed for twenty seconds. After that the solution was centrifuged for three min at 13,000-16,000 rpm until the precipitated proteins formed a tight pellet. The layer, which was containing the DNA, was removed and transferred to 1.5 ml tube containing 600µl isopropanol. Next, the solution was mixed by inversion until thread-like strands of DNA form a visible mass and it was centrifuged at 13,000-16,000 rpm for one min. before the supernatant was transferred to another tube and DNA was washed by adding 600 µl 70% ethanol. The tube containing the mixture was inverted for several times then centrifuged at 13,000-16,000 rpm for one min. Then, the ethanol was aspirated by using either a drawn pasteur pipette or a sequencing pipette tip and the tube was overturned on top of clean absorbent paper and the pellet was air-dried for 15 min. Then, about 100µl of DNA rehydration solution and rehydrate were added to tube and incubated at 65°C for one hour. The solution was mixed periodically by quietly tapping the tube. The extracted DNA was stored in a refrigerator at 2-8°C.

#### 2.4.2. Quantification of DNA

Agarose gel electrophoresis (0.7 %) was used to determine the quality and quantity of the DNA extracted from the *Ziziphora tenuior* L. tissues. The size and the intensity of each sample were detected with DNA mass ladder (Promega) 1 kb to determine genomic DNA concentration.

#### 2.4.3. Amplified Fragment Length Polymorphism (AFLP) Analysis

AFLP profiles were obtained according to (Vos et al., 1995). Six selective primers were used for DNA amplification with two labeled EcoRI (AAC IRDye 700 and ACT IRDye800) and five MseI unlabeled primers (CAC, M-pr-CTT, CGT, CAT and CTC). DNA samples were digested with EcoRI and MseI restriction enzymes and proper oligonucleotide adapters were ligated with DNA ligase through incubation at 37°C for 150 min. Double-stranded adapters were prepared by mixing individual synthetic oligonucleotides: EcoRI-adapter was prepared by mixing 7.0 µL of the top strand oligonucleotide (2µg/µL) with 7.5 µL of the bottom strand oligonucleotide (2µg/µL) in 486.1 µL of TE buffer. MseI adapter was also prepared by mixing 63.5 µL of the top strand oligonucleotide (2µg/µL) with 54.5 µL of the bottom strand oligonucleotide (2µg/µL) in 382 µL of TE buffer.

A pre-amplification step was performed using primers (E-pr-A and M-pr-C) designed to amplify the DNA fragment between the adapter sequence and one additional nucleotide. For quality assessment 10 µL of the reaction was used to run on 1.5% agarose gel and the rest (10 µL) was diluted with 40 µL of TE (10 mM Tris and 1mM EDTA, (pH 8), which was enough for about 30 AFLP-reactions. The diluted reaction was stored at -20°C. Products of pre-amplified DNA were selectively amplified by using combinations of six primers EcoRI+MseI (E-ACC 700 with M-CTC, M-CTA, and M-CAG) and (E-AAG 800 with, M-CTC, M-CTA, and M-CAG).

A touchdown program was used for performed of PCR: thirty cycles of subsequently lowering the annealing temperature (65 °C) by 0.7 °C per cycle while keeping denaturation at 94°C for 30 seconds and extension at 72 °C for 60 seconds. This was followed by 23 cycles of denaturation at 94°C for 30 seconds, annealing at 56 °C for 30 seconds and extension at 72 °C for 60 seconds. After PCR, four µL of blue stop solution were added directly before storage at -20 °C.

#### 2.4.4. Polyacrylamide Gels

Products of AFLP reaction were analyzed by using automated sequencer (LI-COR, Inc. Lincoln, Nebraska, USA). For gel electrophoresis of AFLP, 6% polyacrylamide gel was used, 200 ml polyacrylamide were prepared with 84g urea, 30 ml acrylamide / bisacrylamide 40% solution (SIGMA), 20 ml of 10X Trisborate-EDTA (TBE) buffer. Gels were cast at least 60 minutes before use and were focused by pre-run for 25 minutes before loading. As recommended by LI-COR, the pre-running and running electrophoretic conditions were performed at 1500V, 40W, 40 mA, 45 °C, and 4 scan speed. TBE buffer (1X, 89 mM Tris, 89 mM borate, and 2.2 mM EDTA pH 8.3) was used as the running buffer. The purpose of pre-running was to “warm up” the gel to about 45 °C, this temperature was maintained throughout electrophoresis, resulting in even heat distribution and good quality fingerprints. Samples of DNA were denatured by heating the resulting mixtures for 5 min at 95 °C and cooled rapidly on ice before loading the gel. To remove urea precipitate or pieces of gel, gel wells were completely rinsed prior to loading by flushing them with buffer using a 20 cc syringe, then one µL of each denatured sample was loaded in the designated lanes, IRDye 700 and IRDye 800 sizing standard were used to determine band size.

#### 2.5. Experimental Design

In cryopreservation experimental part, all treatments in each experiment were arranged in a Complete Randomized Design (CRD) and 40 shoot tips were used in each treatment. The collected data from the experiments described above were statistically analyzed using SAS 9.2 (Statistical Analysis System, Cary, NC) and the standard error of means were calculated for each treatment in each cryopreservation experiment.

### 3. Results and Discussion

#### 3.1. Encapsulation-Dehydration

A complete survival of the encapsulated non-cryopreserved *Ziziphora tenuior* L. shoot tips was recorded when shoot tips pretreated with 0.5 M sucrose after dehydration periods of (0, 2 hrs) under laminar air, moisture content of beads MC % ranged from (85%) and (68 %), respectively, for each period of dehydration (Table 1). The recovery percentages in the non-cryopreserved shoot tips showed the greatest values (100, 95%) after (0, 2 hrs) dehydration periods, while the minimum value was recorded after (6 hour) dehydration when the MC% reached to (21%) (Table 1). Similar findings were obtained by Younes (2012) who obtained

the full survival percentage (100%) of encapsulated non-cryopreserved *Achillea fragrantissima* shoot tips in pretreated media provided by 0.5 M sucrose with or without (3 hrs) dehydration when incubated for three days.

Meanwhile, the results of the present study revealed that, after cryopreservation, the highest survival percentage (35%) in shoot tips pretreated with 0.5 M sucrose supplemented media for one day was obtained after (6 hrs) dehydration period before LN exposure, while the maximum recovery percentage (20%) was obtained after (4 hrs) dehydration period with MC% of (25.5%). Also, it was found that, beads with MC % of (85%) and (68%) did not recover after (0, 2 hrs) dehydration (Table 1). Obtaining the best recovery at MC level of (25%) is expected due to the fact that, most of plant species explants recovered after cryogenic treatment when their MC was between 17-37% (Englemann, 2004).

Also, the results of the present study recorded (40 %) survival rate in the cryopreserved *Ziziphora tenuior* L. shoot tips recorded when pretreated with on 0.75 M sucrose provided media for one day and air dehydrated for 6 hrs. Also, the best recovery rate (20%) in the cryopreserved explants was obtained on shoot tips pretreated with either 0.5 or 0.75 M sucrose supplemented media for one day followed by 4 or 6 hrs air dehydration as shown in Fig. (1) and Table (2). However, our results agree with Younes (2012) who reported that the encapsulated shoot tips of *Achillea fragrantissima* obtained the greatest survival and recovery percentages (40, 20%) when pretreated in media provided by 0.5 M sucrose with (6 hrs) dehydration, but it disagrees with her regarding the best recovery which was obtained in her study after incubation for three days, which is not the case in our study. Meanwhile, Moges, et al. (2004) reported that a complete survival of encapsulated cryopreserved violet (*Saintpaulia ionantha*) shoot tips was obtained when pretreated with 0.3 M sucrose and incubated for two days with (2 hrs) of dehydration. Wang et al. (2002) reported that the greatest recovery of encapsulated shoot tips of *Troyer citrange* was obtained when shoot tips precultured in media provided by different concentrations of sucrose ranged from (0.15 M to 0.29 M). In addition, poor growth was obtained when sucrose concentrations in pretreated media lower than (0.15 M or higher than 0.29 M). Maria et al. (2012) reported that promoting recovery of encapsulation cryopreserved white mulberry (*Morus alba*) shoot tips with pretreated media contains 0.75 M sucrose was more effective than 0.5 M sucrose for either one or three days . Also, Shibli et al. (2009) reported that pretreated *Crocus hyemalis* and *Crocus moabiticus* calli gave the greatest survival and recovery percentages, respectively, when encapsulated with 0.5 M sucrose for two days after (2 hrs) of dehydration and cryopreserved. However, the decline in the survival and recovery rates of *Ziziphora tenuior* might be attributed to the formation of extra-cellular and intra cellular- ice crystals as a result of high moisture content of the cryopreserved tissues (Baghdadi et al., 2010; Plessis et al., 1993).

The moisture content of the encapsulated *Ziziphora tenuior* beads declined with increasing exposure duration to air drying (0, 2, 4, 6 hrs), as shown in (Tables 1-4), and

shoot tips with high moisture content were not able to recover after cryogenic exposure (Tables 1-4). High moisture content was found to cause death of the encapsulated African violet (*Saintpaulia ionantha*) shoot tips that pretreated with 0.1 M sucrose for 2 days before (8 hrs) of dehydration (Moges et al., 2004). Also, Tahtamouni and Shibli (1999) showed that moisture content of wild pear beads pretreated with 0.75 M sucrose declined more than those pretreated with 0.3 M sucrose when exposed to the same dehydration period. Survival and recovery were higher of encapsulated cryopreserved bitter almond (*Amygdalus communis*) shoot tips when dehydrated for 6 hrs than 4 hrs (Al-Ababneh et al., 2003). Moreover, (Halmagyi and Deliu, 2011) found that in redwood (*Sequoia sempervirens* Endl.) shoot tips moisture content was reduced to (27%) when pretreated for (24 hrs) in media supplemented with 0.5 M sucrose followed by (3 hrs) air dehydration which resulted in (67%) recovery percentage. Also, greatest recovery after (1.5 or 2 hrs) of dehydration with (33% or 28%) moisture contents of olive shoot tips obtained, respectively (Martinez et al., 1999). However, the reduction in the obtained survival and recovery percentage in the current study (Tables 1-4) might be attributed to partial damage of the shoot tips due to osmotic shock after rehydration and ice crystallization of some cells in the calli (Al-Ababneh et al., 2003), or might be due to unfavorable growth condition (Al-Ababneh, 2002; Moges et al., 2004). This leads to a conclusion that each plant species has its own needs for optimum dehydration to obtain best recovery after cryopreservation, and these needs must be optimized for osmotic dehydration pretreatment in terms of concentration and duration in addition to air dehydration duration.

There were other related studies in Jordan in which the cryopreservation was applied successfully on some medicinal and wild plants. For example, Sharaf et al. (2012) reported a complete survival and (27%) recovery were obtained when encapsulated non-cryopreserved *Artemisia herba-alba* shoot tips were pretreated in 0.5 M sucrose for three days. Also, Shibli et al. (2009) showed that the greatest survival (83.3; 88.9%) and recovery (77.6; 83.3%) percentages were obtained when encapsulated non-cryopreserved calluses of *Crocus hyemalis* and *Crocus moabiticus* precultured with 0.1 M sucrose for two days without dehydration, respectively. A similar result was reported by Moges, et al. (2004) who found that the full survival and the greatest recovery (75%) percentage were obtained when encapsulated non-cryopreserved African violet *Saintpaulia ionantha* shoot tips were pretreated with 0.3 M sucrose for two days with (2 hrs ) air dehydration. Also, Al-Ababneh et al. (2003) reported the best survival and recovery rates in encapsulated cryopreserved bitter almond *Amygdalus communis* shoot tips air dehydrated for 6 hrs.

**Table 1:** Survival and recovery percentage of encapsulated shoot tips of *Ziziphora tenuior* L. as affected by air dehydration duration after pretreatment with 0.5 M sucrose concentration for one day

Sucrose Conc. (M)	Dehydration Duration (hr)*	Survival %	Recovery %	MC %
Non- cryopreserved shoots tips (-LN)				
	0	100* ± 0.0	100* ± 0.0	85
0.5 M sucrose	2	100 ± 0.0	95 ± 3.49	68
	4	90 ± 4.80	75 ± 6.93	25.5
	6	80 ± 6.40	70 ± 7.33	21
Cryopreserved shoots tips (+LN)				
	0	0.0 ± 0.0	0.0 ± 0.0	85
0.5 M sucrose	2	15 ± 5.71	0.0 ± 0.0	68
	4	30 ± 7.33	20 ± 6.40	25.5
	6	35 ± 7.63	15 ± 5.71	21

\*Values represent means ± standard error of mean.

**Table 2:** Survival and recovery percentage of encapsulated shoot tips of *Ziziphora tenuior* L. as affected by dehydration duration after pretreatment with 0.75 M sucrose concentration for one day

Sucrose Conc. (M)	Dehydration Duration (hr)*	Survival %	Recovery %	MC %
Non- cryopreserved shoots tips (-LN)				
	0	100 *± 0.0	100* ± 0.0	80.20
0.75M sucrose	2	95 ± 3.49	90 ± 4.80	51.10
	4	80 ± 6.40	65 ± 7.63	23.00
	6	75 ± 6.93	60 ± 7.84	19.00
Cryopreserved shoots tips (+LN)				
	0	0.0 ± 0.0	0.0 ± 0.0	80.20
0.75M sucrose	2	15 ± 5.71	0.0 ± 0.0	51.10
	4	30 ± 7.33	10 ± 4.80	23.00
	6	40.0 ± 7.84	20 ± 6.40	19.00

\*Values represent means ± standard error of mean.

**Table 3:** Survival and recovery percentage of encapsulated shoot tips of *Ziziphora tenuior* L. as affected by dehydration duration after pretreatment with 0.5M sucrose concentration for three days

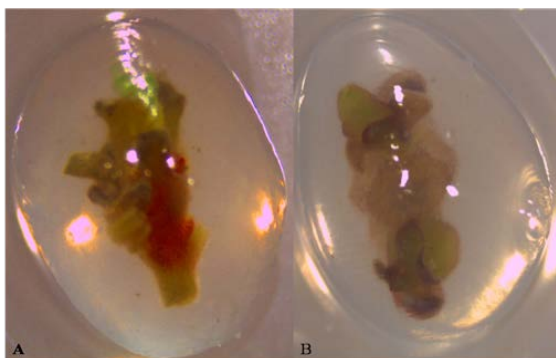
Sucrose Conc. (M)	Dehydration Duration (hr)*	Survival %	Recovery %	MC %
Non- cryopreserved shoots tips (-LN)				
	0	100 *± 0.00	100* ± 0.00	75.8
0.5 M sucrose	2	100 ± 0.00	100 ± 0.00	61.5
	4	90 ± 4.80	85 ± 5.71	26.8
	6	80 ± 6.40	70 ± 7.33	20
Cryopreserved shoots tips (+LN)				
	0	0.0 ± 0.00	0.0 ± 0.00	75.8
0.5 M sucrose	2	20 ± 6.40	0.0 ± 0.00	61.5
	4	30 ± 7.33	0 ± 0.00	26.8
	6	35 ± 7.63	10 ± 4.80	20

\*Values represent means ± standard error of mean.

**Table 4:** Survival and recovery percentage of encapsulated shoot tips of *Ziziphora tenuior* L. as affected by dehydration duration after pretreatment with 0.75M sucrose concentration for three days

Sucrose Conc. (M)	Dehydration Duration (hrs)	Survival %	Recovery %	MC %
Non- cryopreserved shoots tips (-LN)				
	0	100 *± 0.00	100* ± 0.00	70.30
0.75M sucrose	2	95 ± 3.49	90 ± 4.80	55.90
	4	75 ± 6.93	50 ± 8.00	18.10
	6	70 ± 7.33	42.5 ± 7.91	17.80
Cryopreserved shoots tips (+LN)				
	0	0.0 ± 0.00	0.0 ± 0.00	70.30
0.75M sucrose	2	0.0 ± 0.00	0.0 ± 0.00	55.90
	4	20 ± 6.40	0.0 ± 0.00	18.10
	6	10 ± 4.80	0.0 ± 0.00	17.80

\*Values represent means ± standard error of mean.



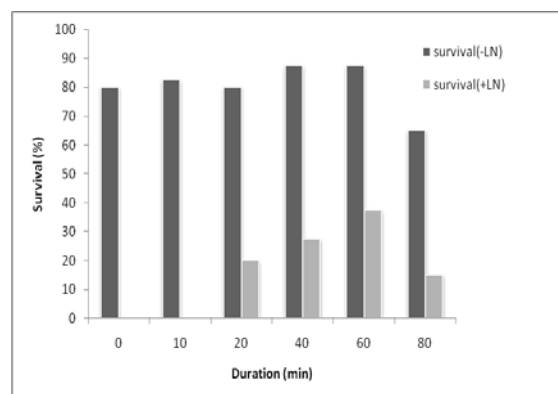
**Figure 1:** Survival and recovery of the cryopreserved encapsulated shoot tips of *Ziziphora tenuior* pretreated with 0.75 M sucrose MS supplemented media for one day followed by 6 hrs. air dehydration under the laminar air-flow cabinet. A: Survival, B: Recovery

### 3.2. Encapsulation-vitrification technique

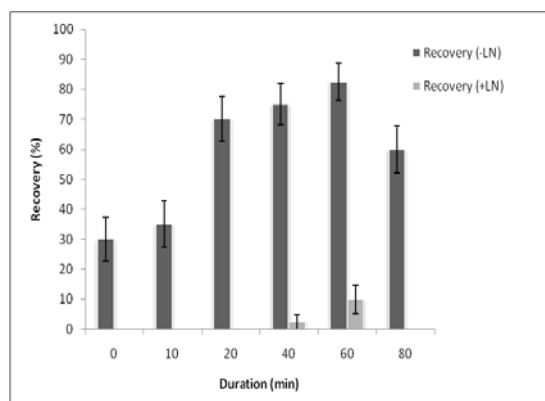
Recovery percentages varied the encapsulated-vitrified shoot tips either with or without (LN). The greatest recovery rates (82.5%, 75%) were recorded in none cryopreserved shoot exposed to the loading solution for either (40 or 60 min) compared to (30%) recovery obtained in unloaded shoot tips (0 min) as shown in the (Figure 2). Younes (2012) reported that the shoot tips of non-cryopreserved *Achillea fragrantissima* gave the greatest survival and recovery percentages when encapsulated shoot tips were pretreated with the loading solution for 80 min. Dumet *et al.* (1993) reported that the increase in survival and recovery percentages of the non-cryopreserved shoot tips in oil palm was due to reduced osmotic shock as shoot tips were subjected to loading solution for longer duration before exposure to PVS2.

Moreover, after the cryopreservation of *Ziziphora tenuior* L. shoot tips, the greatest survival rate (37.5%) was obtained in (60 min) loading duration treatment, while shoot tips in (0, 10 min) duration treatments were not able to record survival (Figures 2- 3). Also, after cryopreservation applied to shoot tips of *Ziziphora tenuior* L. the cryopreserved shoots failed to recover in

most treatments except in (40, 60 min) as (2.5, 10%) recovery rates were recorded, respectively (Figures 2- 3). However, the results obtained for recovery were very low as the highest recovery recorded was only (10%) (Figure 3) which could be attributed to intracellular ice crystals formation during freezing and or which is considered highly harmful on explants (Vandenbussche and Proft, 1996).



**Figure 2:** Survival percentages of non-cryopreserved (-LN) and cryopreserved (+LN) of *Ziziphora tenuior* L. shoot tips as influenced by loading duration with the loading solution using Encapsulation-Vitrification technique. Values represent means ± standard error of mean



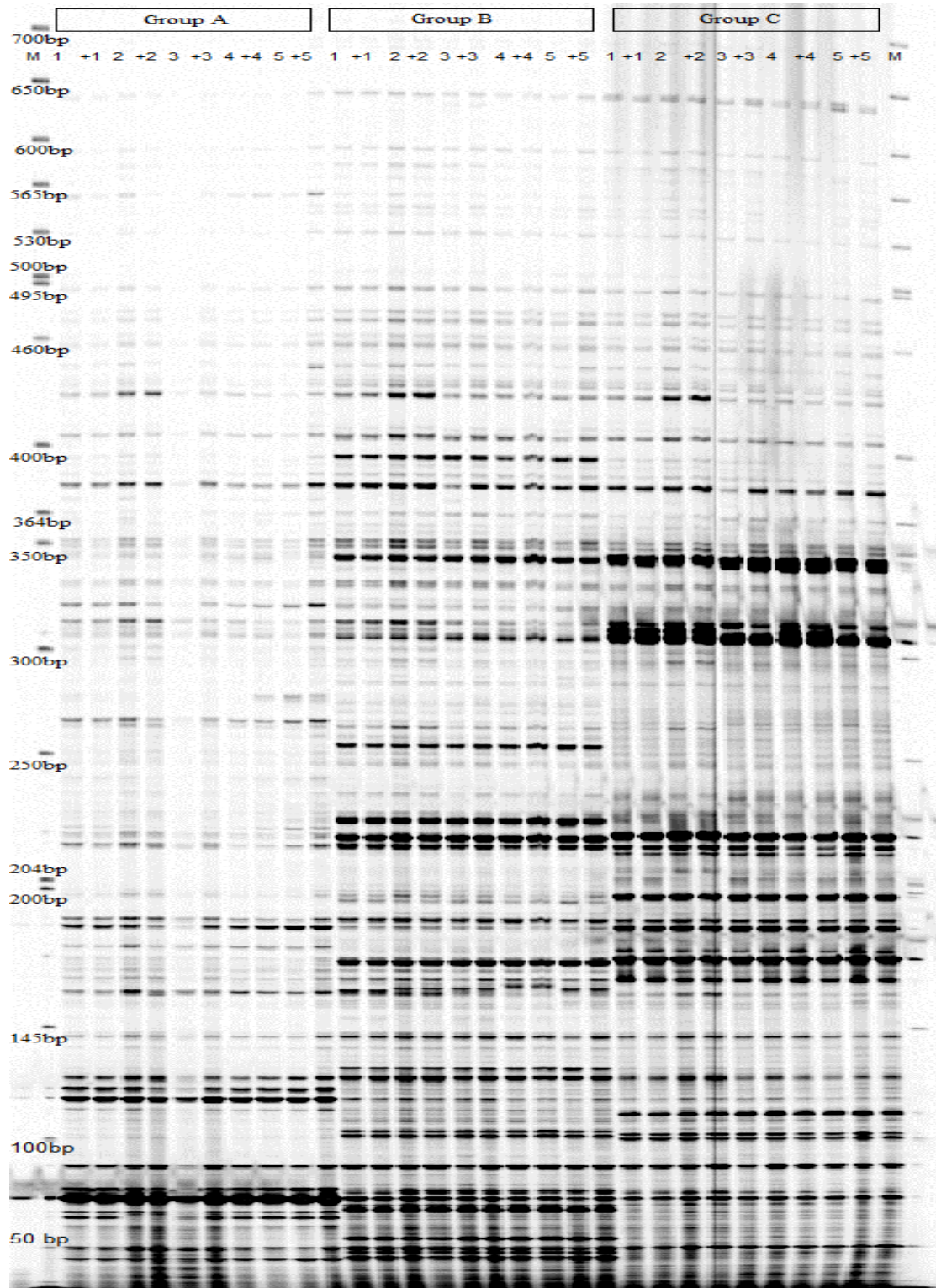
**Figure 3:** Recovery percentages of non-cryopreserved (-LN) and cryopreserved (+LN) of *Ziziphora tenuior* L. shoot tips as influenced by loading duration with the loading solution using Encapsulation-Vitrification technique. Values represent means ± standard error of mean

### 3.3. Genetic Stability

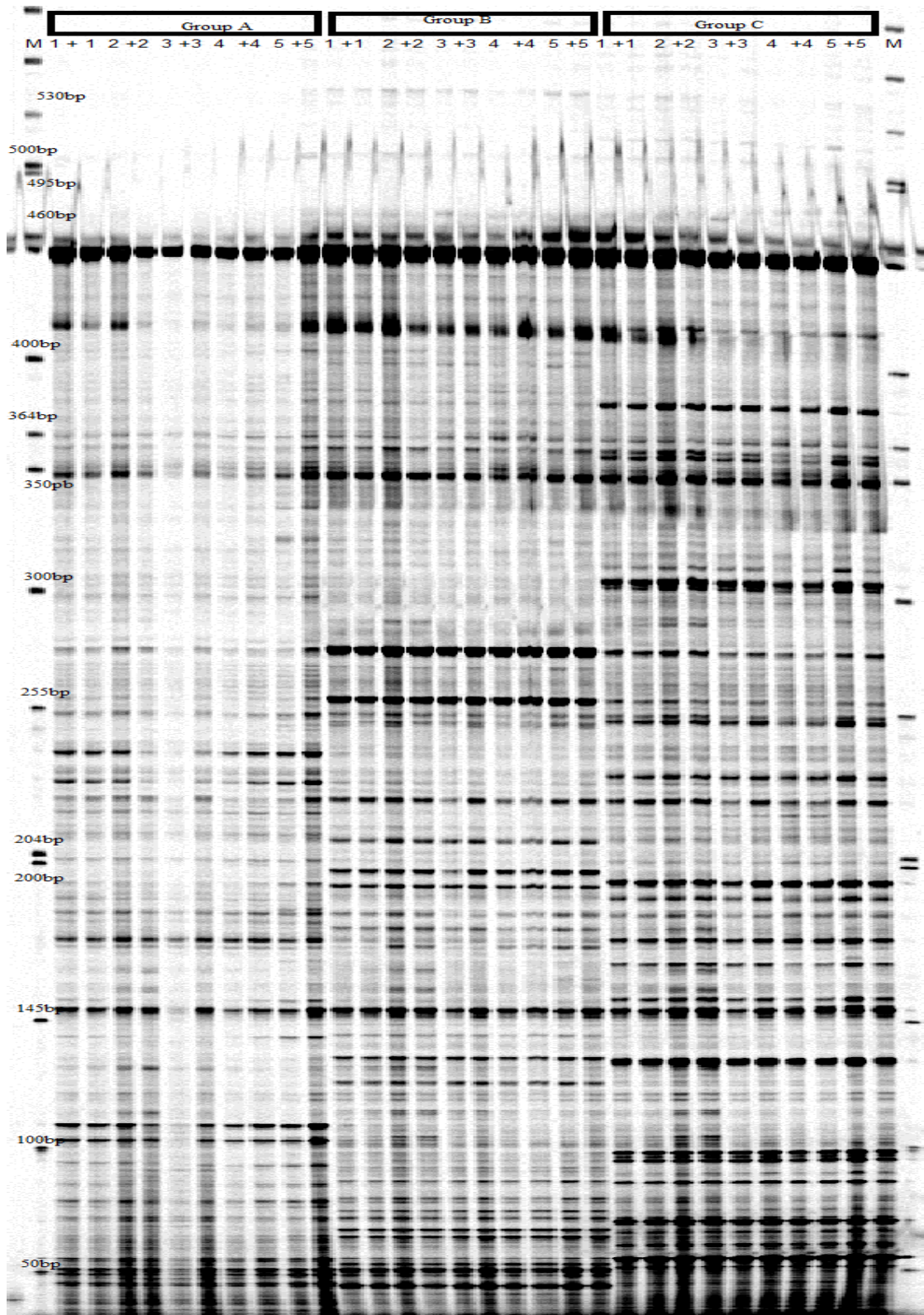
For genetic stability study on *Ziziphora tenuior* L., tissues cryopreserved and non-cryopreserved tissues (Figures 4-5) showed no clear differences when studied by AFLP. This was expected due to the fact that organized tissues (shoot tips) were used in the current study, and only undifferentiated tissues like callus and cell suspension cultures were reported to show some epigenetic variation after exposure to LN (Harding and Benson, 2001). Our results agreed with the previous studies, for example. Castillo *et al.* (2010) found no morphological differences between cryopreserved greenhouse-grown *Rubus grabowskii* when compared with the control mother plants. Lurswijidjarus and Thammasiri (2004) reported that orchid plantlets cryopreserved showed normal growth characteristics after

regrowth. Also, no variations were obtained in the morphology or growth profiles between cryopreserved and non-cryopreserved cell cultures of tobacco (Kobayashi et al., 2006). Studies of Harding (1991) and Harding and Benson (2001) on cryopreserved shoot tips

of (*Solanum tuberosum* L.) confirmed that the stability of the ribosomal RNA genes and the nuclear-chloroplast DNA and the plants exhibited normal developmental patterns after re growth.



**Figure 4:** Amplified DNA patterns using AFLP primers for *Ziziphora tenuior* L., Pre-amplified DNA products were selectively amplified using three *EcoRI*+*MseI* primer combinations (E-ACC 700 with M-CTC (group A), M-CTA (group B), and M-CAG (group C). (+) stands for cryopreserved samples. Numbers from 1-5 represent cryopreserved samples of plants, respectively.



**Figure 5.** Amplified DNA patterns using AFLP primers for *Ziziphora tenuior* L., Pre-amplified DNA products were selectively amplified using three *EcoRI*+ *MseI* primer combinations (E-ACC 800 with M-CTC (group A), M-CTA (group B), and M-CAG (group C). (+) stands for cryopreserved samples. Numbers from 1-5 represent cryopreserved samples of plants, respectively



#### 4. Conclusions

Encapsulation-dehydration in addition to encapsulation-vitrification methods were used for cryopreservation of *Ziziphora tenuior* L. shoot tips. In the encapsulation-dehydration experiment, the best recovery rate (20%) was obtained when either 0.5 M or 0.75 M sucrose MS supplemented media were used as preculture media for the shoot tips for one day, followed by air dehydration for 4 or 6 hrs. Meanwhile, when encapsulation-vitrification experiment was applied, the highest recovery (10%) percentage was obtained when the encapsulated shoot tips were pretreated for 60 min. with the loading solution before exposed to PVS2 vitrification solution and LN. Also, as it was expected, no genetic differences were found in the cryopreserved explants before and after exposure to LN. However, further studies on the in vitro conservation should be initiated to improve recovery rates after cryopreservation.

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